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TOXICOLOGIC AND ANALYTICAL STUDIES WITH T-2 AND RELATED TRICHOTHECENE MYCOTOXINS

FIRST ANNUAL PROGRESS REPORT

AUTHOR(s)
William B. Buck, Project Director
Val R. Beasley, Project Coordinator
Steven P. Swanson, Project Coordinator

March, 1983

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-82-C-2179

College of Veterinary Medicine
University of Illinois
Urbana, Illinois 61801

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T-2 toxin intravascularly produced a cardiovascular shock syndrome in swine characterized by early decreased cardiac output and urine production, and a later decline in blood pressure. Core body temperature remained normal but extremities became cold. Hemoglobin of arterial blood was oxygen saturated in spite of cutaneous cyanosis. Severe metabolic acidosis developed. There were marked elevations in plasma 6-keto-$PGF_1\alpha$ and thromboxane B_2 (metabolites of vasoactive prostanoids), and transitory elevations in epinephrine and norepinephrine. Serum calcium and glucose declined while phosphorus, BUN, magnesium and potassium increased. Pathologic changes included

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20. Abstract (Con'd)

lymphoid necrosis, gastroenteric mucosal hyperemia, enterocyte necrosis, focal myocardial degeneration and endocardial hemorrhage.

A gas chromatographic-electron capture detection method for the analysis of T-2 toxin and metabolites in plasma and urine has been developed. Isolates of Fusarium have been obtained and are being screened for trichothecene profiles. In vitro incubations of T-2 toxin with swine liver microsomes has resulted in the production of several new metabolites.

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SUMMARY OF PROGRESS

I. Swine Pathophysiology Study

Groups of swine have received intravascular doses of 5.4 (preliminary group), 4.8, 0.6 and 0.0 mg/kg T-2 toxin in 70% ethanol. The shock syndrome was characterized by an immediate drop in cardiac output which continued to decrease thereafter. Blood pressure was initially maintained, but began to decline within a few hours. Heart rates were unchanged at first, but subsequently increased. The skin of animals receiving the higher doses became cyanotic in spite of normal oxygenation of arterial blood, and the core body temperature remained relatively normal while the extremities became cold to the touch. A severe metabolic acidosis developed which exceeded the capacity of the animal to compensate by hyperventilation.

There was no evidence of a coagulopathy in animals receiving 5.4 and 4.8 mg/kg T-2 toxin. Similarly, there was no evidence of disseminated intravascular coagulation. Total serum calcium and glucose concentrations declined, while inorganic phosphorus and blood urea nitrogen became elevated. Serum magnesium and potassium increased as did the activities of SGOT and serum alkaline phosphatase. Neutrophilia and lymphocytosis were present in the first hours after administration of T-2 toxin, but subsequently the absolute numbers of these cells fell below predosing values. There were large peaks in plasma epinephrine and norepinephrine concentrations. The metabolites of prostacyclin and thromboxane A_2, 6-keto-PGF_1 and T_xB_2 respectively, showed marked elevations.

Lesions included grossly evident red mucous membranes of the gastric fundus, jejunum and ileum. Microscopically, the mucous membranes of the stomach and intestine were extremely congested and enterocyte necrosis was present. Focal myocardial degeneration was

evident. Hemorrhages were encountered on the endocardial surface of the left ventricle and were seen in the cortical regions of some lymph nodes and intestinal mucosa. The most dramatic microscopic lesion found was necrosis of lymphoid tissue in all parts of the body.

II. Metabolism of T-2 Toxin

Tritium labeled T-2 toxin has been prepared for use in metabolism studies. In vitro metabolism studies with T-2 toxin using swine liver microsomes have been initiated. The major in vitro metabolite, identified by TLC using sulfuric acid and nitrobenzylpyridine color reagents in addition to thin layer radiochromatography, was HT-2. Minor amounts of other trichothecenes were also identified including: neosolaniol, T-2 tetraol, 4-deacetylneosolaniol, and several cmpounds which have not yet been identified.

III. Analytical Methods Development/Residue Analysis

Methods for the detection and quantitation of T-2 toxin and known metabolites in plasma and urine have been developed. The method involves extraction of trichothecenes with XAD-4 followed by cleanup on gel permeation and florisil columns. After derivitization to the heptafluorobutyryl ester derivatives, the toxins are quantitated by gas chromatography with electron capture detection. Analysis of limited plasma and urine samples from swine administered T-2 toxin have shown HT-2 to be the major metabolite.

IV. Trichothecene Toxin Production

Several isolates of toxigenic Fusaria have been obtained from various parts of the world. We have selected Fusarium tricinctum NRRL 3299 as our first T-2 toxin producer. This isolate has been

grown on two substrates under different culture conditions. Culture extracts have been analyzed by capillary gas chromatography for trichothecenes and aliquots of these extracts have been sent to USARMIID for laboratory animal toxicity testing. Screening of additional toxigenic Fusarium isolates is in progress. Limited quantities of known T-2 metabolites have been prepared for use as standards.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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LIST OF INVESTIGATORS

Name	Position/Specialty	Responsibility
William B. Buck	Professor of Toxicology Department of Vet Biosciences	Project Director
Val Richard Beasley	Research Associate, Toxicology Department of Vet Biosciences	Project Coordinator Pathophysiology Studie in Swine
Steven P. Swanson	Senior Research Chemist, Toxicology Department of Vet Biosciences	Project Coordinator, Metabolism, Analysis a Methods Development, a Toxin Production
Murli Manohar	Associate Professor, Cardio- vascular Physiology, Department of Vet Biosciences	Cardiophysiology in Sw
Wanda M. Haschek-Hock	Associate Professor of Pathology Department of Vet Pathobiology	Gross and microscopic Pathology; Cardio- pulmonary pathology
Joseph Simon	Professor of Pathology Department of Vet Pathobiology	Consultant in Patholog
Walter E. Hoffmann	Associate Professor of Clinical Pathology. Depart- ment of Vet Pathobiology and Veterinary Clinical Medicine	Consultant in Clinical Pathology
Stanley E. Curtiss	Professor of Animal Science Environmental Management/ Toxic Gases	Consultant in Inhalati Studies
Christine M. Parks	Postdoctoral Research Assoc/ Cardiovascular Physiology, Department of Vet Biosciences	Cardiovascular Physiol in Swine
Roseanne Lorenzana	Research Associate, Toxicology Department of Vet Biosciences	Pathophysiology in Swine; Clinical Pathology
Richard Corley	Graduate Research Asst, Toxicology/Chemistry; Depart- ment of Vet Biosciences	Metabolism, Analytical Methods, and Product
Victor F. Pang	Research Associate, Pathology Department of Vet Pathobiology	Gross and Microscopic Pathology

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Richard K. Manuel	Laboratory Manager/Toxicology Department of Vet Biosciences	Coordination of Personnel and Laboratory Studies
Arthur M. Siegel	Graduate Research Assistant/ Vet. Med. Department of Vet Biosciences	Data Analysis and Computer Programming
Richard Pfeiffer	Research Associate/Toxicology-Chemistry; Department of Vet Biosciences	Methods Development and Production
Catherine A. Knupp	Graduate Research Assistant/ Toxicology-Chemistry; Department of Vet. Biosciences	Trichothecene Analyses
William C. Kisseberth	Graduate Research Assistant/ Toxicology Department of Vet Biosciences	Pathophysiology Studies in Swine
John J. Ciribassi	Graduate Research Assistant/ Toxicology Department of Vet Biosciences	Pathophysiology Studies in Swine
Charles Purdy	Research Technician/Toxicology-Chemistry; Department of Vet Biosciences	Production and Analysis
Dean Rood	Technician, Toxicology-Chemistry; Department of Vet Biosciences	Production and Analysis
Donald White	Associate Professor, Department of Plant Pathology	Fusarium Culturing and Toxin Production
Brian Anderson	Graduate Research Assistant Department of Plant Pathology	Fusarium Culturing and Toxin Production

RESEARCH PROGRESS -- July 1, 1982 through March 10, 1983

I. Research Accomplished Under Study I -- Swine Pathophysiology

A. Objectives and Introduction

Swine were chosen as a suitable model for human exposure to T-2 toxin because the cardiovascular system and general physiology are acceptable for comparison to man. We have previously demonstrated that T-2 toxin, administered in sufficient doses by either intravascular in intragastric routes causes a shock syndrome. One primary objective of this study is to assess the nature of T-2 induced shock using intravascular and inhalation routes of administration. To accomplish this goal, we investigated the effects of intravascular T-2 toxin with regard to: functional alterations in the cardiovascular and pulmonary systems, the appearance of endogenous vasoactive mediators, as well as changes in hematology, coagulation assays and serum enzyme and clinical chemistries.

Another objective in this research project is to compare lesions of T-2 toxicosis by intravascular and inhalation routes of administration. These gross pathology and histopathology findings are, at this point, preliminary as the tissues of the low dose and control animals (given the vehicle only) are currently being processed. Inhalation dosing is scheduled to being in April.

A final objective in this project is to investigate therapeutic agents in T-2 toxicosis. To evaluate agents of potential use, a preliminary study in rats was performed after a lethal intraperitoneal dose of T-2 toxin was determined. T-2 toxin at

this dosage was given to four groups. Group A was pretreated with a preparation of selenium and vitamin E; Group B with aspirin; and Group C with dexamethasone. Group D received no other treatment. The variable of interest was the change in percent lethality as compared to the nontreated group.

B. Preliminary and Preparatory Work

Procurement of the appropriate equipment and supplies was initiated for blood, serum and plasma assays. To establish a range of normal porcine values, and to refine and standardize experimental protocols, blood, serum and plasma samples from normal swine were collected. Evaluation of these specimens included assessment of: hematology, platelet counts, activated clotting times, fibrin degradation products assays, prothrombin and partial thromboplastin times, catecholamines, and histamine and serotonin concentrations. The collection of these specimens also provided an opportunity for training of research personnel.

Commercial laboratories were employed for analysis of plasma for catecholamines, histamine and serotonin. In order to provide samples representing the range of anticipated alterations expected in the definitive study, an LD_{50} dose of T-2 toxin (1.2 mg/kg) was given intravascularly to one pig.

In the preliminary group (Group 1, n=2), catheters were surgically placed in the aorta, pulmonary artery and left atrium. After a 3 week recovery period, T-2 toxin was administered intravascularly at 5.4 mg/kg. This was done in order to assess the appropriateness of the cardiovascular instrumentation and method of restraint, in acute T-2 toxicosis. These initial studies also

allowed us to gain preliminary blood, plasma and serum values in order to assure that we would be within linear ranges on assays of importance. The results of administration of a highly lethal dose to this group permitted tailoring of procedural protocols, identification of appropriate assays and selective focus on parameters of noticeable alteration in subsequent groups.

The clinical and gross post mortem similarities between shock induced by T-2 toxin and by endotoxin lead to an investigation of the role of prostacyclin (PGI_2) and thromboxane (TxA_2). Consequently, lethal doses of endotoxin were given arterially to two swine to obtain plasma samples to be used in validation of a commercially available assay kit for the metabolites of PGI_2 (6-keto-prostaglandin F_1) and TxA_2 (thromboxane B_2), and to provide positive control samples. Samples of plasma from preliminary Group 1 animals revealed increases in 6-keto-prostaglandin F_1 (6-keto PGF_1) and thromboxane B_2 (TxB_2) and therefore the same assays were performed in the formal study.

C. Formal Study

1. High dose of T-2 toxin given intravascularly

a. Cardiovascular and pulmonary functional alterations

All pigs used in these studies had normal cardiac output and blood pressures and were active and alert before dosing. The formal study began with the adminisration of a high dose of T-2 toxin (4.8 mg/kg) to five swine via the pulmonary artery over 6 minutes using an infusion pump. These are referred to as Group 2 (n=5) in this report. In the assessment of cardiovascular and

pulmonary function the following were measured every thirty minutes post injection of T-2 toxin: heart rate, aortic mean pressure, aortic systolic pressure, aortic diastolic pressure, pulmonary artery mean pressure, left atrial mean pressure, cardiac output, arterial pH, arterial pCO_2, arterial pO_2, hemoglobin content, animal temperature, and hemoglobin percent saturation.

Table 1 gives a summary of changes seen in these animals. In this group, cardiac output began to decline at fifteen to thirty minutes after administration of T-2 toxin, and was reduced by approximately 25% at one hour. At each successive hour, cardiac output declined by 10-15% of the previous reading until death occurred. It was noted that cardiac output decreased before a reduction in systemic pressure occurred, as reflected in the mean aortic pressure.

In this group, left atrial mean pressure remained normal throughout the entire experiment, indicating that ventricular filling was unchanged and not a factor in the decreased cardiac output. Mean pulmonary artery pressure was stable or slightly increased. This circumstance coincident with decline in cardiac output indicated pulmonary vasoconstriction and increased intrapulmonary pressure.

These data seem to indicate that 4.8 mg/kg T-2 toxin given intravascularly has an immediate detrimental effect

on the heart as well as on systemic vasculature, resulting in progressive hypotension and inadequacy of circulation. Compensation was attempted, as indicated by an increased heart rate, but was not successful.

Table 2 gives mean values obtained from Group 2. Arterial blood pH declined and arterial pCO_2 decreased or stayed the same. This correlated clinically with hyperventilation as the animals unsuccessfully attempted to compensate for the acidosis. This clearly suggested a metabolic acidemia which overwhelmed the body's buffering systems. With the concurrent decrease in cardiac output, an increase in anaerobic metabolism is suspected with resultant production of lactic acid. Uric acid may also be produced if ATP is being completely degraded. We are presently refining our method for measurement of lactic acid and selecting an appropriate technique for uric acid determination. These areas will be more fully investigated in the second year.

Body temperature of T-2 treated swine remained near normal in the face of tremendous systemic shock until just prior to death. Apparently central homeostatic control mechanisms were not directly affected by T-2 toxicosis. It may be noted that these studies were conducted at ambient (indoor) temperatures and whether central thermoregulation would be maintained under more stressful circumstances is uncertain.

In the T-2 treated pigs of Group 2, hemoglobin content increased as did the hematocrit (Table 2). The concentration of hemoglobin is a direct indication of the oxygen carrying capacity of blood. This increase in hemoglobin probably reflects splenic contraction and dumping of red blood cells into the circulatory system. Splenic smooth muscle is sensitive to stimulation by catecholamines. The data in Table 3 (in the section on vasoactive mediators) verified a rise in circulating catecholamines.

In Table 3 it can be observed that both epinephrine and norepinephrine substantially increased suggesting stimulation of the adrenal medulla. In Table 4, it is pointed out that epinephrine is not secreted by sympathetic nerve endings. This data suggests involvement of both the autonomic and central nervous system in development of T-2 toxicosis.

Prostacyclin (PGI_2) is continuously produced by vascular endothelial cells and has a blood half-life of three minutes. It is a potent peripheral and coronary vasodilator, has antiaggregation and anti-deposition activity in platelets, and increases bleeding time. Prostacyclin is also produced in the renal cortex where it is believed to interact with the renin-angiotension system.[2] By contrast thromboxane A_2 (TxA_2) with a half-life of only three seconds, has opposite effects. It aggregates platelets and constricts smooth muscle. The

balance between these two hormones may participate in maintenance of vascular integrity and imbalances may occur during shock.[3] In addition, prostacyclin may have a central affect in cardiovascular regulation.[4,5]

The compounds, 6-keto-PGF_1 and TxB_2 are the sole degradation products of PGI_2 and TxA_2, respectively. An assay for these metabolites has been demonstrated to be appropriate for the detection of their short-lived parent compounds. Commercially available radioimmunoassay kits (New England Nuclear Corp., Boston, Mass.) were validated for use on unextracted porcine plasma.

Figure 1 and Table 5 show the concentrations of 6-keto-PGF_1 and TxB_2 in Group 1. Figure 2 and Table 6 give the values for Group 2. It is seen that both hormones reach high plasma concentrations and that TxB_2 rises before 6-keto-PGF_1. Thromboxane A_2 has been called a shock inducing agent. This substance has been shown to produce coronary and arterial vasospasm, and can lead to myocardial ischemia and myocardial infarction.[3]

These data suggest that T-2 toxin in some way stimulates release of these prostanoids.

b. Vasoactive Mediators

In three T-2 dosed swine no variation in histamine or serotonin levels occurred, although a shock syndrome developed. Therefore, we have discontinued monitoring for these substances concluding that they do not contribute to the toxic syndrome brought on by T-2 toxin.

In contrast, catecholamines rose to high levels. Table 3 shows the mean values for norepinephrine, epinephrine and dopamine in swine given 4.8 mg/kg of T-2 toxin intravascularly. There is a wide range in each catecholamine value, therefore interpretation using mean values does not give a fully accurate impression. Individual animals showed wide oscillation between low and high values as shown in Figure 4. This probably reflects the pulsatile release of catecholamines in response to such stresses as the shock induced by T-2 toxin, abdominal pain, vomiting or diarrhea.

The sharp decreases reflect the subsequent rapid metabolism of catecholamines. Norepinephrine and epinephrine are catabolized by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Both are widely distributed. MAO is found in mitochondria of brain, liver, kidney and nerve tissue. Reuptake of norepinephrine is the major mode of clearance of autonomic nerve endings.[1]

We cannot suggest the significance of the circulating levels of dopamine at this time. It is primarily a neurotransmitter in the brain and sympathetic ganglia. In peripheral ganglia dopamine is an inhibiting substance, modulating and regulating transmission. It is also catabolized by MAO and COMT.

c. Serum enzyme and biochemistry

The values for blood urea nitrogen (BUN) and serum creatinine indicated decreased glomerular filtration of these urine products in Group 2 (Table 7). Sodium levels

remained relatively constant and potassium increased but not to magnitudes that would be clinically symptomatic. This correlated clinically with the cessation of urine production in this group; as well as with the decline in systemic blood pressure. (Table 1) Decreased glomerular filtration may have been due to the decline in adequate filtration pressure, to renal vasoconstriction as a result of high levels of circulating catecholamines and thromboxane, or to T-2 toxin or its metabolites if they are directly nephrotoxic.

In Group 2 (Table 7) phosphorus and magnesium levels increased while total calcium and ionized calcium (values not tabulated) moderately decreased. Glutamic pyruvic transaminase (SGPT), glutamic oxalacetic transaminase (SGOT), and alkaline phosphatase (SAP) all increased although the SGPT activity was increased to a lesser extent than that of the other two.

Increases in calcium in the myocardium are associated with ischemia of the myocardium. As calcium in intracellular fluid is present in extremely low concentrations and as calcium is an intracellular modulator that rivals cyclic AMP in importance, this finding is of much interest.

High levels of phosphorus are a prominent cause of acidosis in renal disease. An elevated level also results in a reciprocal decline in calcium.[6] In this group phosphorus is probably a contributor rather than the cause of metabolic acodosis.

Magnesium is the principle cation of soft tissue and is ten times more concentrated intracellularly than extracellularly. Losses of magnesium from the myocardium occur in myocardial ischemia. Serum magnesium levels also increase in renal failure.

SGPT and SGOT are found in the cell sap of hepatocytes. In some species, SGPT is liver specific; however, considerable amounts of SGOT are located within muscle tissue. SAP is not tissue specific and is found in liver, bone, intestine and kidney where it is associated with the cell membrane and microsomes. The rise in these intracellular and membrane constituents suggests disruption of cellular membranes.

Blood glucose increases at the second and third hour, then declines well below predosing levels. The increase is most likely a stress response comprised of a release of adrenal corticosteroids which induce gluconeogenesis. The decline of blood glucose may be a result of catecholamine induced glycolysis, the blockade of gluconeogenesis, or increased utilization of glucose. The decline in serum glucose will be confirmed in subsequent animals.

d. Coagulation

Along with the presence of other parameters, the appearance of fibrin degradation products and reduced circulating platelet numbers has been used to substantiate the occurrence of disseminated intravascular

coagulation (DIC). DIC is said to occur in many different types of shock syndromes (e.g.: endotoxic shock) and is a consumptive coagulopathy leading to abnormal hemorrhage.[7]

Assay for fibrin degradation products (FDP) was carried out on samples from six animals given T-2 toxin intravascularly. Blood samples were collected hourly. No FDP's were identified in any of the samples. In addition, the number of circulating platelets (Table 8) did not decline.

Review of the mean values of prothrombin time (PT), partial thromboplastin time (PTT) and activated clotting time (ACT) on Table 8 reveals no evidence of prolongation of these parameters.

From these data, it can be concluded that 4.8 mg/kg T-2 toxin given intravascularly to swine does not result in any acute coagulation deficiency. Microscopically evident congestion, which was so severe that it is grossly indistinguishable from hemorrhage, has been seen in the gastric and intestinal mucosa, meninges and heart; however this suggests a vascular effect by T-2 toxin. (See also section on histopathology.) In other studies hemorrhage has been found in areas of necrosis.

e. Hematology

Mean values of hematologic parameters of Group 2 are shown in Table 9. Obvious trends seen in the leukogram are graphically represented in Figure 3. The total

white blood cell numbers increase to the levels seen at the first hour, then rapidly decline. This rise is due to an absolute increase in the number of segmented neutrophils and lymphocytes and indicates a stress response with shift of cells from marginal storage pools. By 3-4 hours after administration of toxin the numbers of neutrophils and lymphocytes fall below predosing levels. A common cause of lymphopenia is the release of endogenous corticosteroids in response to stress. The neutrophilic response to stress, however, is toward an increased circulatory half-life and therefore an increase is seen in mature cells. This response is not reflected in this data. In addition, a bone marrow response is absent as evidenced by the lack of significant increase in immature neutrophils (band cells).

The deficiency in the number of white blood cells may indicate destruction, increased utilization, sequestration, extravasation and/or reduced production. Destruction of band cells normally stored in the bone marrow could be substantiated if the necrotic debris seen in the bone marrow were a result of granulocytic loss (see section describing histopathology). This could explain the abnormally low numbers of circulating band cells. There is evidence of sequestration in the spleen and liver (see section on histopathology), but, there is, as yet, no evidence for increased utilization or extravasation.

The effects on the total and differential white blood counts are to be reviewed with our consulting clinical pathologist.

f. Pathology

Summary of gross findings in Group 2 (n=5):

The snout and the skin over the abdomen were slightly to moderately purple. The lateral aspect of the left apical and cardiac lung lobes was tightly adhered to the thoracic wall in each pig. There was approximately 40-50 ml of a straw colored clear fluid in the thoracic cavity of one pig. Adhesion was noted between the pericardiac sac and the epicardium in 4 pigs. Five hundred mls of blood tinged fluid was present in the pericardiac sac of the remaining pig. Areas of moderate to severe subendocardial hemorrhage were seen in the left ventricle, especially in the area adjacent to the mitral valve.

The peripheral and visceral lymph nodes were moderately swollen, mottled red and slightly to moderately edematous. The spleen was soft and the white pulp was not as prominent as normal.

The mucosa of the gastric fundus was diffusely dark red to purple and was covered by a layer of thick mucus. The mucosa of the ileum, especially in the terminal portion, was diffusely red. The content in the small intestine and spiral colon was semifluid. There were no formed feces in the terminal colon.

The liver was mottled red. The wall of the gall bladder was severely edematous. Occasionally, petechial hemorrhages were present on the survace of the adrenal gland and the parenchyma was mottled red. The mucosa of the uterus was red. The meninges were congested, and in some cases there were large bright red areas grossly indistinguishable from hemorrhages. Occasionally, the thyroid gland was mottled red.

Summary of micropathology findings in Group 2 (n=5):

Thymus -- There was mild to moderate lymphoid depletion and a small to large number of tingible body macrophages in the cortex. Mild to moderate lymphocytic necrosis was noted in the medulla with some small eosinophil aggregates randomly present.

Palatine tonsil -- Diffuse severe lymphocytic necrosis, mainly in the germinal centers was accompanied by areas of mild to moderate neutrophil infiltration. Multifocal, mild to severe degeneration and necrosis occurred in the cryptal epithelium. A moderate to large number of transepithelial lymphocytes were becoming degenerative and necrotic.

Submandibular, cervical, mediastinal, bronchial, mesenteric and inguinal lymph nodes -- There was diffuse moderate to marked lymphocytic depletion and lymphocytic necrosis, primarily in the germinal centers. Mild to moderate reticuloendothelial hyperplasia, and areas of edema and small neutrophil aggregates were seen in the sinuses and

were accompanied by areas of hemorrhages, primarily in the subcapsular region.

Spleen -- Diffuse moderate to severe lymphocytic necrosis and lymphoid depletion were observed in the white pulp with scattered mild perifollicular hemorrhage. The red pulp was moderately to severely congested with mild to moderate leukocytosis (neutrophils) in the sinuses.

Stomach -- Areas of epithelial sloughing were present on the mucosal surface. Diffuse, moderate to marked congestion, moderate edema and occasional mild hemorrhage were noted in the luminal phase of the lamina propria. Occasionally, fibrin thrombi were seen in capillaries. Necrosis was observed in the small lymphoid nodules in the submucosa and in the lymphocytes in the lamina propria.

Intestine -- Mild to severe villous necrosis, primarily in the small intestine was observed. Congestion, occasional mild hemorrhage and fibrin thrombi were also present. Moderate to severe cryptal necrosis was diffusely present, especially in jejunum and ileum. The number of mononuclear cells in the lamina propria was moderately increased throughout the intestine. Severe necrosis was noted in the lymphocytic population. Prominent necrosis was present in Peyer's patches and lymphoid nodules throughout the submucosa.

Gall bladder -- Moderate to severe subserosal edema was present.

Liver -- Areas of mild to moderate congestion and sinusoid leukocytosis (neutrophils and lymphocytes) are noted. Occasionally, there was a mild neutrophil and eosinophil infiltration in the perilobular fibrous tissue. The hepatocytes were slightly swollen.

Pancreas -- Occasionally, individual cell degeneration and necrosis were present in the islets of Langerhans.

Lung -- The bronchus associated lymphoid nodules and the peribronchiolar and perivascular lymphoid aggregates were moderately to severely necrotic. Areas of congestion were noted in the interalveolar septa.

Heart -- Diffuse subepicardial fibrosis, edema and neovascularization were noted with minimal mononuclear cell infiltration. Areas of mild to severe congestion and hemorrhage occurred in the subendocardial myocardium. Focal hyalinization and fragmentation were present in the affected muscle bundles.

Kidney -- Scattered mild fibrosis and mononuclear cell infiltrates were seen in the interstitium. Cellular necrosis was noted in the mononuclear cell infiltrates and subpelvic lymphoid nodules. Congestion was present in the glomeruli and interstitium.

Adrenal -- Individual cell degeneration and necrosis were randomly distributed in the cortex. Mild leukocytosis was present in sinusoids.

Ovary -- Degeneration and necrosis occurred in the ova of primary follicles.

It was determined that 1.25 mg/kg T-2 toxin was suitable to observe the effect of therapeutic pretreatments.

Each group consisted of eleven male rats of 149-293 grams in weight. Group A received 0.1 mg/kg of selenium, intramuscularly, at 72, 48 and 24 hours before administration of T-2 toxin. Selenium was chosen for its ability to enhance levels of the enzyme glutathione peroxidase. The enzyme has a half-life of approximately 21 days. The time interval of pretreatment was chosen to insure that enzyme induction had occurred. The preparation used[a] also contained vitamin E; consequently 2 mg/kg of this vitamin was also given.

Group B received 150 mg/kg acetylsalicylic[b] acid orally 48 hours, 24 hours and just prior to administration of T-2 toxin. It has been demonstrated that 3 mg/kg of aspirin in adult humans will inhibit platelet production of TxA_2 for the life of the platelet.[9] By interpolation this may represent approximately one third of the analgesic therapeutic dose. In rats the therapeutic dose of aspirin is 450 mg/kg;[10] therefore 150 mg/kg was chosed for this study.

Group C received 4 mg/kg dexamethasone[c] intramuscularly at 48 hours, 24 hours and just prior to administration of T-2 toxin. The plasma half-life of dexamethasone

---

[a] E-Se^R, Burns Biotec, Omaha, NB

[b] Aspirin, USP powder, Humco Lab, Texarkana, TX (100 mg/ml in 70% EtOH)

[c] Dexate^{TM}-SP, Burns Biotec, Omaha, NB

is approximately 3-4 hours but the anti-inflammatory affect is approximately 72 hours. The principle reasons for choosing this pretreatment were for the gluconeogenic affect and ability to stabilize membranes. Membrane stabilization would have two consequences of value here: to prevent release of lysosomal enzymes and to reduce release of membrane bound fatty acids that are converted by phospholipases to endoperoxides. Endoperoxides are precursors of PBI_2 and TxA_2. An additional argument for the use of corticosteroids is their inhibition of the release of ACTH from the anterior pituitary. B-lipotropin a precursor of B endorphin, originates from the same molecule as ACTH. It is believed B-lipotropin release is inhibited along with ACTH by corticosteroids. B-endorphin has recently been implicated in the pathophysiology of shock.[11]

Table 10 compares the mortality between groups. These percentages suggest that selenium/vitamin E and dexamethasone decrease mortality, but that this dose of aspirin (acetylsalicylic acid) does not give protection. It is interesting that at 8 hours after injection of T-2 toxin, mortality was much lower in the dexamethasone treated group.

The results of the dosing in these few rats is clearly preliminary. Further and more sophisticated investigation is needed to establish an adequate therapeutic regimen.

D. Studies Remaining

Completion of analyses and evaluations of pathological specimens of the low dose and control groups is now in progress. Another group of instrumented, intravascularly dosed swine will be used to determine the myocardial contractility and myocardial blood flow utilizing radiolabeled microspheres and high fidelity-catheter tip transducers. Animal dosing in this part of the study should be completed by the end of April, 1983.

Also during the month of April, we will be working toward finalization of nebulization and particle characterization methodology for T-2 toxin. T-2 toxin grows as a columnar crystal. In our work to produce a crystal less than 5 microns in its greatest dimension, recrystallization in various solvents, grinding, blending and multiple filtration have been the various methods attempted. The resultant particles were measured using scanning electron-photomicrography and were found to exceed the desired size limitation. Recently we have consulted with the Department of Ceramic Engineering at the University of Illinois at Urbana. By their suggestion, we will next utilize low temperature recrystallization in an ultrasonic field, to see whether appropriately sized particles can be produced. If this is unsuccessful T-2 will be nebulized as a solution.

Inhalation studies similar to those using intravascular dosing will then be completed.

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TABLE 1-1

Group 2 -- Individual's summary of cardiovascular and pulmonary function
4.8 mg/kg T-2 toxin, intravascular

	Predosing Values			1 Hour Postdosing			2 Hours Postdosing			4 Hours Postdosing			6 Hours Postdosing			Time of Death
Animal Number	Heart Rate	Aortic Mean Pressure	Cardiac Output l/min	Heart Rate	Aortic Mean Pressure	Cardiac Output l/min	Heart Rate	Aortic Mean Pressure	Cardiac Output l/min	Heart Rate	Aortic Mean Pressure	Cardiac Output l/min	Heart Rate	Aortic Mean Pressure	Cardiac Output l/min	Hours Postdosing
211	123	118	10.74	108	120	7.57	186	96	5.52	228	52	3.39				4.75
213	117	*	10.07	105	*	7.58	114	*	7.34	138	72	6.04	150	56	5.28	10.5
215	128	120	8.56	156	118	8.03	144	98	9.31	168	46	6.18	168	40	5.25	6.5
217	132	134	11.34	132	124	8.51	138	116	8.43	216	72	5.41	246	58	4.10	7.0
221	136	136	9.78	152	128	8.87	162	120	6.82	206	68	6.00	235	52	4.02	7.5

* Catheter not functional

TABLE I-2

Group 2 -- Mean Values of Blood pH and Blood Gases
4.8 mg/kg T-2 toxin, intravascular

Hour	0 n=5	1 n=5	2 n=5	3 n=5	4 n=5	5 n=4	6 n=4	7 n=3	8 n=1	9 n=1	10.5 n=1
Arterial pH	7.435	7.345	7.347	7.289	7.265	7.270	7.257	7.092	7.232	7.207	7.149
Arterial pCO_2	36.6	36.5	37.2	36.9	35.4	35.6	32.8	42.4	33.4	30.5	35.4
Arterial pO_2	90.1	90.5	88.4	83.4	84.9	87.4	86.9	65.1*	102.5	105.6	97.8
Hemoglobin	10.8	12.3	12.8	13.1	12.7	12.0	12.2	13.4	10.9	11.3	13.3
% Saturation of Hemoglobin	100.1	98.5	97.5	96.9	94.0	96.1	95.2	62.0*	96.4	97.5	96.9
Body Temp (^oC)	39.7	39.7	39.7	39.8	40.0	39.6	39.4	40.0	37.7	37.6	37.2

* weighted by low values from animals near death, see Table I-1

TABLE I-3

Group 2 -- Mean Values for Catecholamines
4.8 mg/kg T-2 toxin, intravascular

Hour	0 n=6	1 n=5	2 n=5	3 n=5	4 n=5	5 n=4	6 n=4	7 n=3	8 n=1	9 n=1	10.5 n=1
Norepinephrine pg/ml	790	4729	1910	5515	1815	1083	5245	1640	2778	3724	3122
Epinephrine pg/ml	1119	4778	3135	6958	3595	2140	6228	3055	4284	4098	7856
Dopamine pg/ml	252	175	89	200	118	109	231	198	375	375	

TABLE I-4

Source of Catecholamines

Substance	Location Secreted
Norepinephrine	Most postganglionic sympathetic endings, brain, spinal cord, adrenal medulla
Epinephrine	Brain, spinal cord, adrenal medulla
Dopamine	Sympathetic ganglia, brain, retina

Adapted from Ganong, Review of Medical Physiology, 10th ed.[1]

TABLE 1-7, Page 2

Hours	0 n=5	1 n=5	2 n=5	3 n=5	4 n=5	5 n=4	6 n=4	7 n=3	8 n=1	9 n=1	10.5 n=1
Chloride meq/L	144 ± 3.9	No significant change →									
Potassium meq/L	3.9 ± .5	4.3 ± .4	4.9 ± .7	5.2 ± .7	5.6 ± 1.0	5.4 ± 1.0	5.7 ± 1.0	6.4 ± 1.2	5.7	6.0	6.7
SGPT U/L	84 ± 23	161 ± 164	135 ± 93	102 ± 37	94 ± 25	92 ± 23	104 ± 28	78 ± 44	94	94	94
SGOT U/L	36.7 ± 9.3	39.0 ± 12.8	47.2 ± 11.9	55.0 ± 10.7	65.2 ± 13.7	66.5 ± 11.5	80.25 ± 19.3	84.3 ± 26	87	93	114
SAP U/L	51 ± 10	62 ± 30	68 ± 30	72 ± 25	102 ± 35	96 ± 20	100 ± 14	110 ± 20	126	126	138
Bilirubin mg/dl	0.2 ± 0.05	No significant change →									
Glucose mg/dl	84 ± 10.5	73 ± 27.5	110 ± 19.6	128 ± 46	93 ± 89.5	61 ± 44	38 ± 30	61 ± 32	29	29	27

TABLE I-8

Group 2 -- Mean Values of Bleeding Parameters
4.8 mg/kg T-2 toxin intravascular

Hour	0 n=6	1 n=5	2 n=5	3 n=5	4 n=5	5 n=4	6 n=4	7 n=3	8 n=1	9 n=1	10.5 n=1
PT (secs)	13.2 ± 1.3	12.1 ± 1.1	11.9 ± 1.3	12.2 ± 1.3	13.6 ± 3.1	11.9 ± .3	12.9 ± .4	13.3 ± .7	15.3	15.9	16.8
PTT (secs)	23.7 ± 7.7	18.9 ± 5.2	19.4 ± 5.9	19.5 ± 6.1	21.0 ± 6.2	21.3 ± 4.9	20.6 ± 4.7	20.5 ± 8.0	----	----	----
ACT (secs)	108 ± 12.1	105 ± 15.8	99 ± 8.2	95 ± 6.1	112 ± 23.6	93 ± 8.5	93 ± 11.0	98 ± 17.6	95	95	110
Platelet per ul	396166	44000	375400	310266	384800	425750	399333	363000	397000	323000	236000

PT = prothrombin time

PTT = partial thromboplastin time

ACT = activated clotting time

TABLE I-9

Group 2 -- Mean Values of Hematologic Parameters
4.8 mg/kg T-2 Toxin, intravascular

Hour	0 n=5	1 n=5	2 n=5	3 n=5	4 n=5	5 n=4	6 n=4	7 n=3	8 n=1	9 n=1	10.5 n=1
PCV %	33.4 ± 4.7	36.0 ± 5.2	39.0 ± 7.5	39.6 ± 5.5	36.7 ± 5.7	36.5 ± 3.8	33.5 ± 3.5	38.0 ± 4.2	34.0	35.0	----
Hb g/dl	10.8 ± 1.4	12.3 ± 1.8	12.8 ± 2.1	13.1 ± 1.9	12.6 ± 1.8	11.9 ± 1.5	11.4 ± 2.1	12.4 ± 1.9	10.9	11.3	13.3
RBC $1x10^6$/ul	7.59 ± 1.15	8.33 ± 1.48	9.45 ± 1.54	8.98 ± 1.24	9.25 ± 1.36	8.11 ± 1.12	7.95 ± 1.19	8.85 ± 2.36	7.63	8.03	9.50
WBC $1x10^3$/ul	22.6 ± 5.7	34.2 ± 6.7	23.4 ± 8.6	19.1 ± 8.7	13.1 ± 9.1	7.8 ± 2.1	9.6 ± 3.2	10.0 ± 3.5	14.7	15.5	10.5
Abs. Seg $1x10^3$/ul	8.47	15.48	8.05	5.79	3.32	0.86	2.09	3.09	5.59	6.51	7.05
Abs. Lym $1x10^3$/ul	12.52	17.39	14.69	12.70	9.18	6.54	6.50	6.13	7.94	8.53	8.69
Abs. Bands $1x10^3$/ul	3.36	0	0	1.85	3.06	0.63	5.65	3.00	----	----	----
Abs. Mono $1x10^2$/ul	6.90	5.24	2.43	0.54	0.45	0	0	0	0	0	0
Abs. Bas $1x10^2$/ul	3.78	1.29	0.48	0.54	0.83	1.04	0.28	0	4.41	0	0
Abs. NRBC $1x10^2$/ul	0	0.002	0.006	0.006	0.028	0.056	0.153	0.250	----	----	----

TABLE I-10

Mortality of Rats Receiving 1.25 mg/kg
T-2 Toxin Intraperitoneally

	% Mortality	
	At 8 Hours	At 24 Hours
No treatment n=11	64	91
Selenium + Vitamin E n=11	45	64
Dexamethasone n=11	9	82
Aspirin n=11	55	100

Figure I-1

Group 1 mean values of 6-keto-PGF_1 and TxB_2 in plasma, 5.4 mg/kg T-2 toxin, intravascular.

Figure I-2

Group 2 mean values of 6-keto-PGF_1 and TxB_2 in plasma, 4.8 mg/kg T-2 toxin, intravascular.

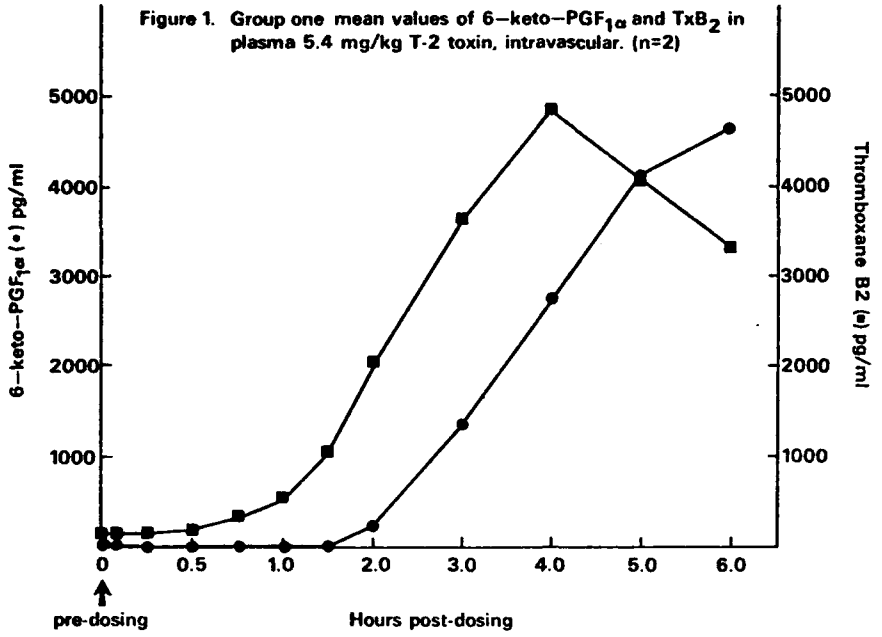


Figure 1. Group one mean values of 6—keto—$PGF_{1\alpha}$ and TxB_2 in plasma 5.4 mg/kg T-2 toxin, intravascular. (n=2)

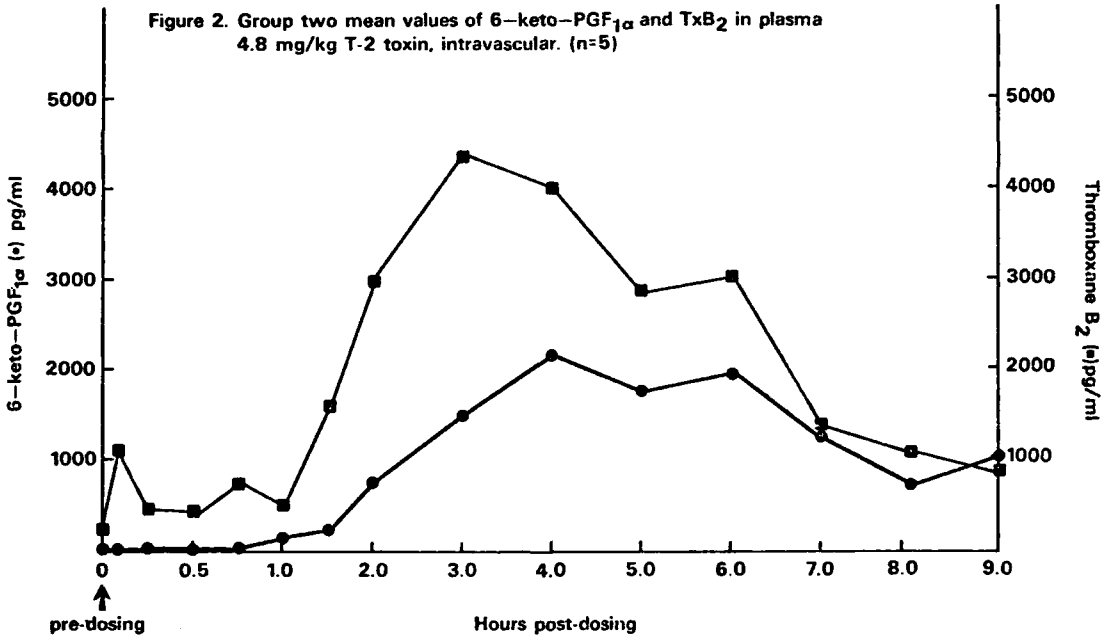
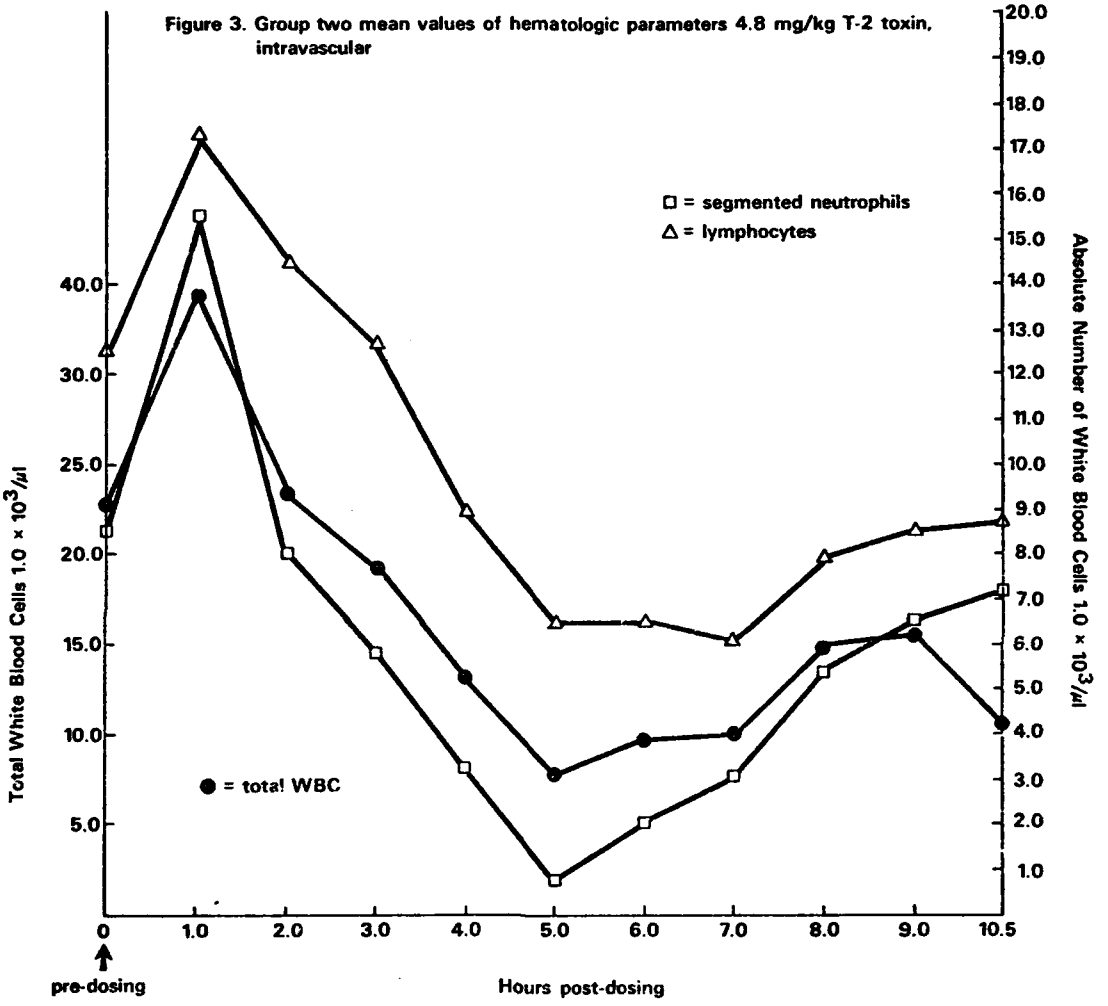


Figure 2. Group two mean values of 6—keto—$PGF_{1\alpha}$ and TxB_2 in plasma 4.8 mg/kg T-2 toxin, intravascular. (n=5)

Figure I-3

Group 2 mean values of hematologic parameters, 4.8 mg/kg T-2 toxin, intravascular.

Figure 3. Group two mean values of hematologic parameters 4.8 mg/kg T-2 toxin, intravascular



II. Metabolism of T-2 Toxin

A. Objectives of this study are:

1. Prepare sufficient quantities of tritium labeled T-2 toxin for animal studies.

Results:

We have prepared tritiated T-2 toxin with a specific radioactivity of 2.6 mCi/mMol for use in metabolism studies. Additional radiolabeled T-2 will be synthesized as needed.

2. Administer tritiated T-2 toxin to swine intravascularly and monitor total radioactivity and metabolic profiles in blood, urine and tissues.

Results:

Administration of radiolabeled T-2 toxin to swine is scheduled for the second half of the first year's investigation.

3. Attempt to isolate and characterize the major metabolites of T-2 toxin <u>in vitro</u> by incubation of tritiated T-2 with swine liver S-9 and microsomal fractions.

Methods:

a. Preparation of Microsomes

Swine liver microsomes were prepared according to the method of Hansen, et al. (1981).[1] The liver was removed immediately following exsanguination, weighed and 100 g were perfused through exposed blood vessels with cold homogenizing medium (0.25 M sucrose + 0.05 mM EDTA) and placed on ice. The liver was diced and homogenized in 3 volumes homogenizing medium and filtered

through glass wool. Microsomes were sedimented from the 10,000 x g supernatant by centrifugation at 100,000 x g for 1 hour. Microsomal pellets were resuspended in homogenizing medium at a final concentration of 1 g liver fresh weight per ml, degased with nitrogen, flash-frozen in dry ice-acetone and stored at -20°C. Microsomes stored in this manner have been showed to retain carbon monoxide spectral binding characteristics for several weeks.[2]

B. Enzyme Incubations

Microsomes were prepared for incubation by mixing microsomes equivalent to 100 mg fresh liver per ml Tris buffer (pH 7.4) plus 0.15 M potassium chloride. Incubations were carried out in 20 ml vials at 37°C using a Dubnoff metabolic shaker. Tritiated T-2 or cold T-2 was added in 0.05 ml ethanol to give a final incubation concentration of 0.1 and 1.0 mM respectively. The reaction was initiated by adding 1 ml of diluted microsomal preparation to 3 ml of Tris buffer-cofactor solution. Final concentrations in 4 ml was as follows: 100 mg liver fresh weight; Tris, 0.05 M, pH 7.4; glucose-6-phosphate, 2.5 mM; glucose-6-phosphate dehydrogenase, 1.33 units; NADP, 0.5 mM; $MgCl_2$, 7.5 mM. Reactions were terminated after 30 minutes by addition of 1 g NaCl to each vial followed by partitioning of metabolites into ethyl acetate. The ethyl acetate extracts from incubation mixtures containing 0.1 mM tritiated T-2 were combined and drained through anhydrous Na_2SO_4 and

concentrated for analysis. The extracts from incubation mixtures containing 1.0 mM cold T-2 were combined in a similar manner. Aliquots of combined tritiated T-2 incubations were spotted on silica gel TLC plates. After development, bands were scraped for analysis of radioactivity by liquid scintillation counting.

We have found that by using high performance TLC (HPTLC) plates with a preabsorbent strip we could monitor the reaction by spotting aliquots of the incubation mixture directly on the HPTLC plate. This has greatly reduced analysis time over conventional methods that require termination of the reaction, extraction and cleanup prior to TLC. Utilizing this technique, 5 microliter aliquots from a selected incubation mixture containing 1.0 mM cold T-2 were spotted on duplicate HPTLC plates at 0, 5, 10, 15 and 30 minutes incubation time. Both plates were developed in methylene chloride-methanol (9+1). One plate was subsequently sprayed with 20% H_2SO_4 in methanol, heated at 120°C for 5 minutes and viewed under long-wave U.V. The other plate was dipped in 3% 4-(p-nitrobenzyl) pyridine (NBP), heated at 150°C for 30 minutes, cooled to room temperature and dipped in 10% tetraethylenepentamine (TEPA) for visualization of trichothecenes. (See Figure 1)

Results

1. Analysis of aliquots spotted directly from 1.0 mM T-2 incubation mixtures.

Two HPTLC plates with preabsorbent strips were spotted directly at time intervals specified in the methods section. Both plates were developed in methylene chloride-methanol (9+1). One plate was treated with H_2SO_4 spray reagent and viewed under U.V. light while the other plate was treated with the NBP-TEPA reagent as described under the methods section. The use of H_2SO_4/U.V. for visualization of compounds by TLC provides excellent sensitivity for many organic compounds although little selectivity is obtained. This high degree of sensitivity has enabled us to visualize several possible metabolites of T-2 that are produced in such small amounts <u>in vitro</u> as to otherwise escape detection. The NBP-TEPA reagent, while not as sensitive as H_2SO_4/U.V., is highly selective for compounds such as trichothecenes by reacting with the epoxide of the trichothecene nucleus. Since stable epoxides are rarely encountered in biological systems this reagent eliminates the need for more rigorous sample preparation prior to analysis. Although absolute identification of a compound is not possible by TLC, the use of such a reagent as NBP-TEPA coupled with the chromatographic properties of known standards provides a good indication that a trichothecene is present. Conditions are currently being developed for identification and confirmation of possible metabolites by capillary gas chromatography-electron capture and flame ionization detection. Additional confirmation will be accomplished by mass spectrometry. Our mass spectrometer has been delivered but not yet installed. The instrument should become available during the summer of 1983.

The results are depicted in Figure 1. HT-2 is produced almost immediately after the reaction is initiated by addition of some

microsomes. No other compound was visualized by NBP-TEPA. Two additional compounds were detected by H_2SO_4/U.V. with R_f values between T-2 and HT-2. From this data we can conclude that HT-2 is the primary metabolite produced in vitro by swine liver microsomes under the conditions given. To detect the presence of other minor metabolites extracts were combined from several incubation mixtures as described in the methods section.

2. Analysis 1.0 mM T-2 incubation extracts

The combined ethyl acetate extracts were concentrated and redissolved in 0.2 ml TLC spotting solvent (3% acetonitrile in benzene). Aliquots of 1, 5 and 5 microliters were spotted on each of two silica gel HPTLC plates. To verify the presence of known metabolites of T-2 one microgram each of T-2, neosolaniol (NEO), HT-2, 4-deacetyl-neosolaniol (4-DN) and T-2 tetraol (Tol) were cospotted with one of the 5 microliter aliquots of incubation extracts. Standards were included on each plate for identification. Both plates were developed in benzene-acetone (3+2). This TLC solvent system was selected over other systems, both normal and reversed phase, since it provides excellent separation of many trichothecene mycotoxins especially the metabolites of T-2, in one development. After development, one plate was treated with the NBP-TEPA reagent while the other plate was treated with H_2SO_4 as described previously.

The results of this analysis indicated the presence of at least 13 compounds in the combined ethyl acetate extracts that can be visualized by H_2SO_4/U.V. and have been assigned code numbers PM-1 through PM-13. Of these 13 compounds 8 were present in sufficient quantities to react with NBP-TEPA to give the characteristic blue

spot, indicating the possible presence of a trichothecene. From this group of 8 compounds visualized by both H_2SO_4/U.V. and NBP-TEPA five were identified as T-2, neosolaniol, HT-2, 4-deacetyl-neosolaniol and T-2 tetraol. A summary of these results are given in Table 1.

3. Analysis of 0.1 mM tritiated T-2 incubation extracts

Concentrated ethyl acetate extracts from tritiated T-2 incubations were redissolved in the TLC spotting solvent. An aliquot was spotted on a silica gel HPTLC plate with preabsorbent strip as well as an aliquot from the tritiated T-2 standard used for the incubations. Three millimeters wide bands were scraped into scintillation vials from the plate after development in benzene-acetone (3+2). To each vial 2 drops of water and 0.5 ml ethanol were added to deactivate the silica gel and desorb the metabolites respectively. Aquasol-2 was used as the scintillation cocktail. Standards and controls were included to determine background and correct for quenching during analysis by liquid scintillation counting.

Results are summarized in Figures 2 and 3. Table 1 correlates radioactivity to compounds visualized by both H_2SO_4/U.V. and NBP-TEPA. From this data we can conclude that several metabolites of T-2 toxin that are presently uncharacterized can be produced by swine liver microsomes. Under the reaction conditions described in the methods section HT-2 is the major <u>in vitro</u> metabolite produced by swine liver microsomes. Of the total radioactivity present on the developed HPTLC plate, 49% corresponded to HT-2 and 43% unmetabolized T-2. Studies are currently underway to optimize conditions for the production of other known as well as uncharacterized metabolites. In addition to

production and characterization of T-2 metabolites, studies are being designed to investigate the involvement of various microsomal enzyme systems through known enzyme inducers and inhibitors.

References

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TABLE II-1

Table 1 Summary of HPTLC analysis of microsomal incubations of T-2 and tritiated T-2

Code No.	Compound[A]	R_f[B]	H_2SO_4[C]	NBP-TEPA[D]	Radioactivity[E]
PM-1		0.88	+	+	-
PM-2		0.86	+	-	-
PM-3		0.79	+	-	-
PM-4		0.65	+	-	+
PM-5	T-2	0.53	+	+	+
PM-6		0.49	+	+	+
PM-7		0.34	+	+	+
PM-8	Neo[F]	0.30	+	-	-
PM-9		0.28	+	-	+
PM-10	HT-2	0.21	+	+	+
PM-11		0.15	+	-	+
PM-12	4-DN	0.11	+	+	+
PM-13	Tol	0.05	+	+	+

A. Identification of compound by HPTLC analysis

B. R_f values for compounds by HPTLC developed in benzene-acetone (3+2)

C. H_2SO_4/U.V. detection, + or -

D. NBP-TEPA detection, + or -

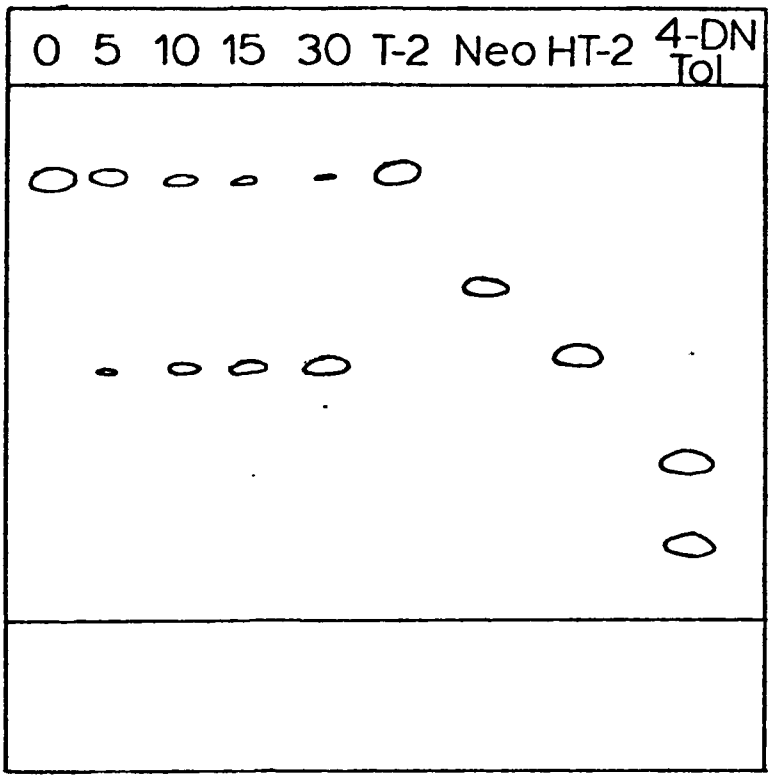
E. Radioactivity present at greater than twice the background, + or -

F. Neosolaniol was only detected in incubations that were allowed to proceed for 2 hours. All other compounds were detected after 30 minutes.

Figure 1

HPTLC plates of aliquote spotted at 0, 5, 10, 15 and 30 minutes time intervals directly from incubation mixtures and developed in methylene chloride-methanol (9+1). Standards of T-2 toxin, Neosolaniol (Neo), HT-2, 4-Deacetylneosolaniol (4-DN) and T-2 tetraol (Tol) at 1 microgram each are included. (A) NBP-TEPA reagent (B) H_2SO_4/U.V.

A)



B)

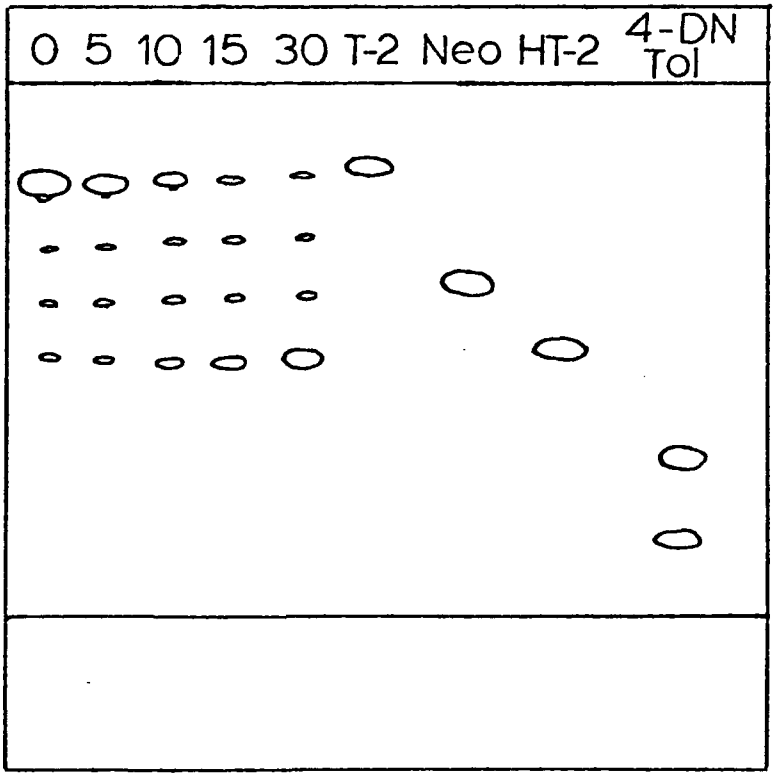


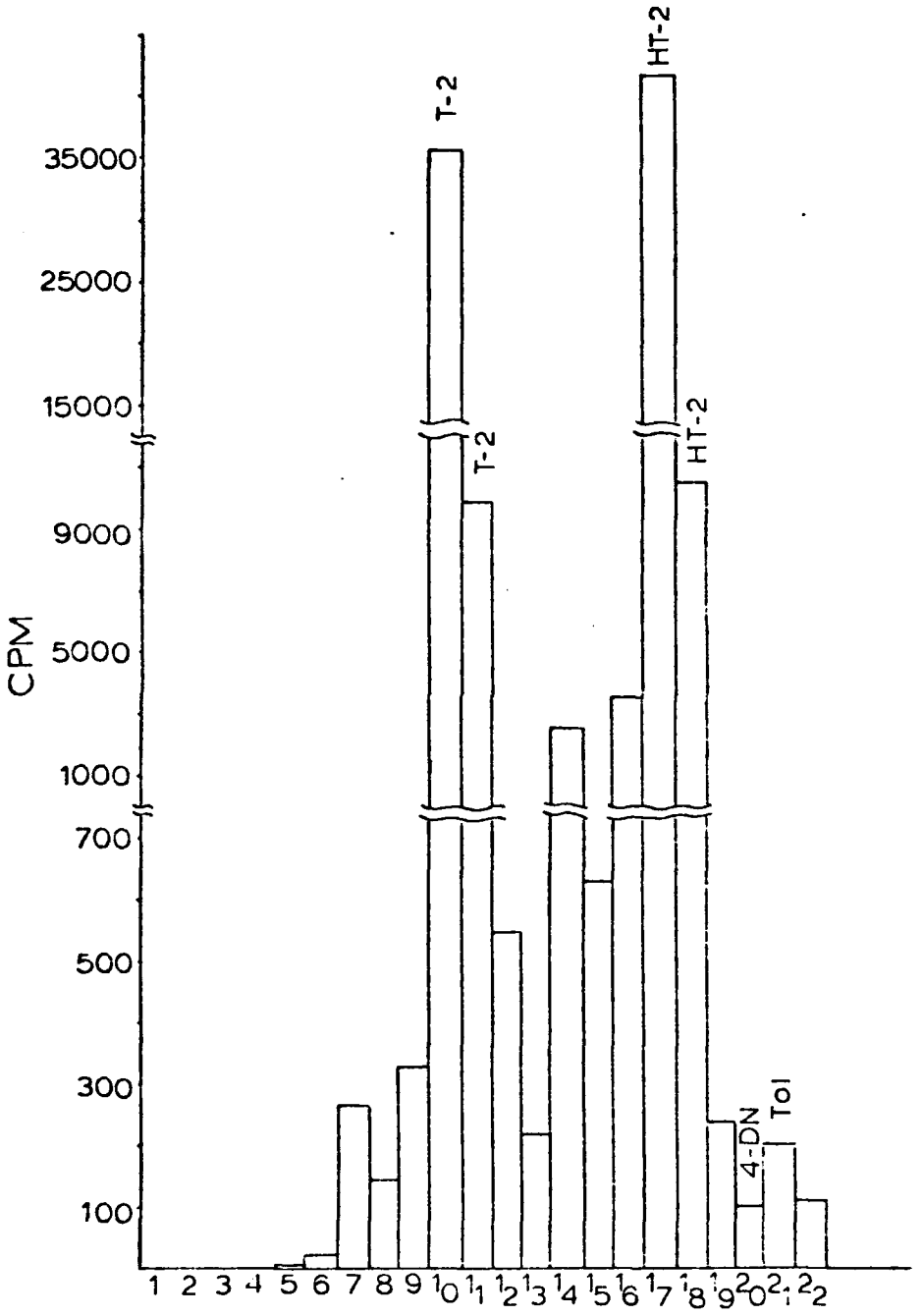
Figure 2

HPTLC plate depicting the position of compounds detected in extracts from microsomal incubations of tritiated T-2 by H_2SO_4/U.V. relative to 3 mm wide bands analyzed by liquid scintillation counting. Nonradioactive standards of T-2, neosolaniol (Neo), HT-2, 4-Deacetylneosolaniol (4-DN) and T-2 tetraol (Tol) are shown for identification. An aliquot of tritiated T-2 standard was included to verify that the radioactive compounds produced by swine liver microsomes were not contaminants of the tritiated T-2 standard (shown on right side of diagram). All values of activity (cpm) are corrected for background and quenching.

CPM Extract	H_2SO_4 / U.V.	CPM (^3H)T-2
0		0
0		0
0		0
0		16
8		0
25		23
265		62
143		70
328		69
35,677		8,043
9,965	T-2	16,523
549		249
220		44
2,519		11
626	Neo	6
3,569		5
41,730		10
10,558	HT-2	18
239		11
103	4-DN	0
201		1
112	Tol	16
	Extract (^3H)T-2	

Figure 3

Histogram of radioactivity (cpm) vs band number scraped from HPTLC plate developed in benzene-acetone (3+2). The 3 mm wide bands are numbered starting at the solvent from (#1) to 1 mm below the origin (#22).


CPM
35000
25000
15000
9000
5000
1000
700
500
300
100
T-2
T-2
HT-2
HT-2
4-DN
Tol
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

III. Methods Development

A. Objectives of this study were:

1. To develop analytical methods which can detect low levels of trichothecenes and metabolites in biological samples.
2. Analysis of samples obtained from swine dosed with T-2 toxin.

B. Procedure

The following procedure has been developed for the analysis of T-2 toxin and metabolites in plasma and urine. Five mL of plasma or urine are diluted with an equal volume of phosphate buffer (10% KH_2PO_4), added to a 1 x 14 cm column of XAD-4 and the flow rate adjusted to 1 mL/minute. The column is rinsed with 50 mL water (flow 3 mL/minute) and the trichothecenes eluted with 100 mL ethylacetate-acetonitrile (9+1). The eluate is drained through a funnel containing 2.5 g fluorouracil and 10 g anhydrous sodium sulfate. After concentration the residue is redissolved in ethyl acetate-toluene (3+1) and injected into an automated ABC gel permeation instrument equipped with a 2.5 x 45 cm column of BioRad SX-3. The 95-120 mL fraction is collected and concentrated. The residue is redissolved in methylene chloride and added to a 2 g fluorouracil column. The column is rinsed with 50 mL methylene chloride-acetone (97+3) and the trichothecenes eluted with 50 mL methylene chloride-methanol (95+5).

After concentration the residue is redissolved in toluene-acetonitrile (95+5), derivatized with heptafluorobutyrylimidazole (HFBI), washed with sodium bicarbonate and diluted with hexane. The final dilution is analyzed by gas chromatography with electron capture detection (ECD).

Results

The XAD-4 column proved to be the best choice for extracting both T-2 and metabolites from plasma and urine. The XAD-4 column was superior to ethyl acetate partitions in both efficiency of extraction and amount of impurities coextracted. Although ethyl acetate readily extracted T-2, the polar trichothecenes (T-2 tetraol, nivalenol) were not effectively extracted even after three partitioning steps.

Several solvents were investigated for elution of trichothecenes from the XAD-4 column including: methanol, ethanol, acetonitrile, acetone and ethyl acetate. Methanol, which is the most commonly reported elution solvent for XAD columns, efficiently eluted the trichothecenes but also coeluted the greatest amount of impurities. Ethyl acetate eluted the least amount of impurities while eluting the trichothecenes with comparable efficiency to methanol. The fluorouracil funnel was added at this step to remove additional impurities and also aid in drying the extract prior to concentration. Addition of 10% acetonitrile to the elution solvent was then necessary to quantitatively elute the toxins from the fluorouracil. The acetonitrile also speeded the concentration by azeotropic distillation of water remaining in the extract. Recoveries of T-2, HT-2, neosolaniol and T-2 tetraol were 100% for the XAD-4 extraction step.

Gel filtration and fluorouracil column chromatography provide additional cleanup, necessary for detection of low ppb levels of toxin. Ethyl acetate-toluene (3+1) was chosen as

the solvent for gel filtration. Methylene chloride-toluene (85+15) and cylohexane-methylene chloride (1+1) solvent systems were also investigated; however, these solvents did not adequately dissolve the polar trichothecenes. Initially two fractions were collected from the fluorouracil column: 1) methylene chloride-acetone (8+2) and 2) methylene chloride-methanol (95+5). Although this provided slightly cleaner extracts more time was required for analysis and HT-2 was unevenly split between the two fractions.

Although isothermal gas chromatography separates the major T-2 toxin metabolites, T-2 tetraol elutes very close to the solvent front and separation of this compound is sometimes obscured by interferences. Capillary gas chromatography with temperature programming and electron capture detection is expected to eliminate interference problems due to the greater resolving power of capillary columns. This technique is currently under investigation. We have recently purchased a gas chromatograph/mass spectrometer (GC/MS). When this instrument has been installed, results from GLC-ECD analyses will be compared with the results obtained by GC/MS.

Even with isothermal TLC this method works well for analysis of plasma and urine. No interferences were detected at the retention times of T-2 and HT-2. See Figures 1 and 2 for chromatograms of standard trichothecenes and a chromatogram of control swine plasma. Recovery experiments are underway. Preliminary results have shown recoveries of T-2, HT-2 and neosolaniol have ranged from 75-110%. At this time we have also

analyzed several samples of plasma from a swine dosed with T-2 toxin intravascularly. See Figure 3 for a chromatogram of a plasma sample taken from a swine dosed with 1.2 mg/kg body weight and blood collected 10 minutes after toxin administration. At this time interval T-2 and HT-2 are the only major toxins detected. A trace amount (3ppb) of neosolaniol was also observed. Further samples of plasma and urine from swine dosed with T-2 toxin is in progress. The majority of samples, however, will not be analyzed until the capillary ECD is set up.

Figure 1

Chromatogram of trichothecene standards (250 pg each). A, Iso T-2 (Internal Standard); B, T-2 toxin; C, HT-2; D, Neosolaniol; E, T-2 tetraol. Compounds were derivatized to their corresponding heptafluorobutyryl esters prior to analysis by gas chromatography with electron capture detection.

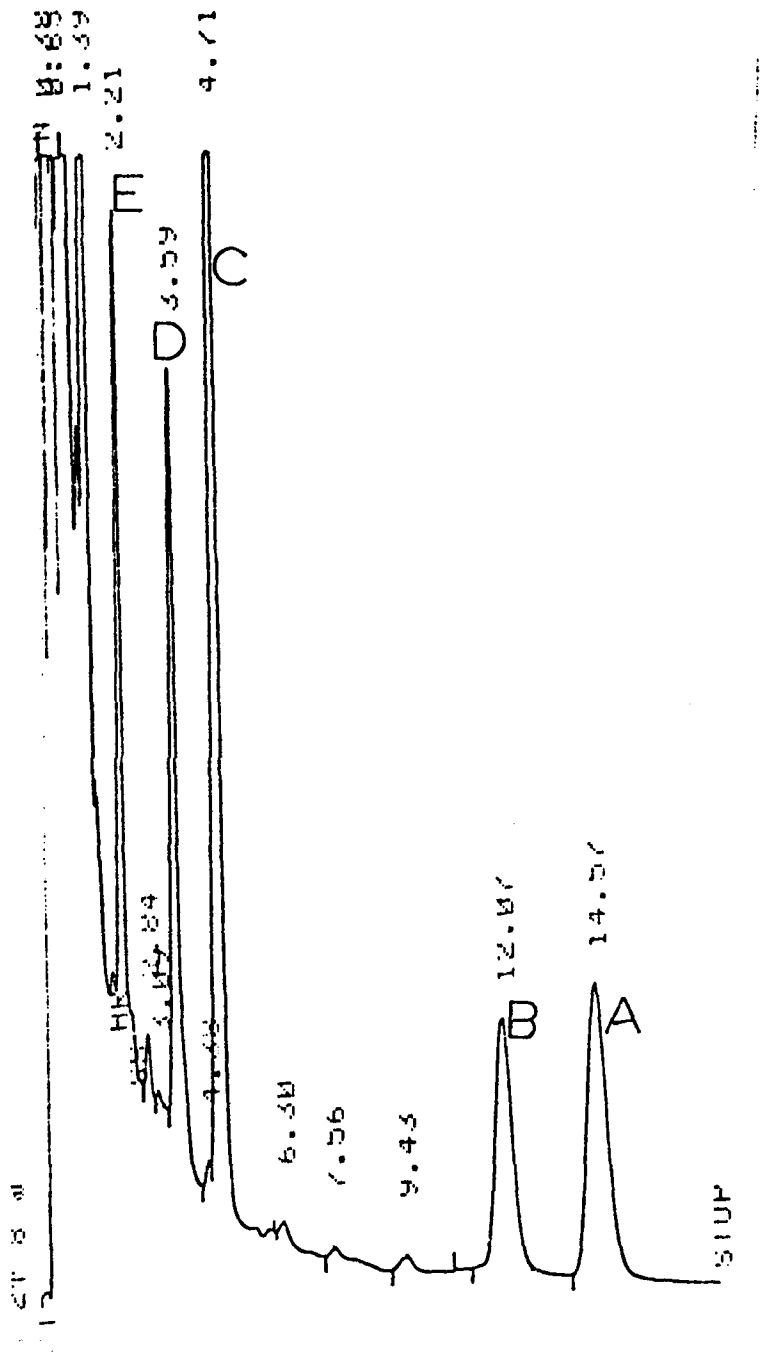

E
D
C
B
A
12.87
14.57
SOLVENT

Figure 2

Chromatogram of control pig plasma, 1.3 mg equivalents injected. Letters indicate retention time of trichothecene: A, Iso T-2 (Internal standard); B, T-2 toxin; C, HT-2; D, Neosolaniol.

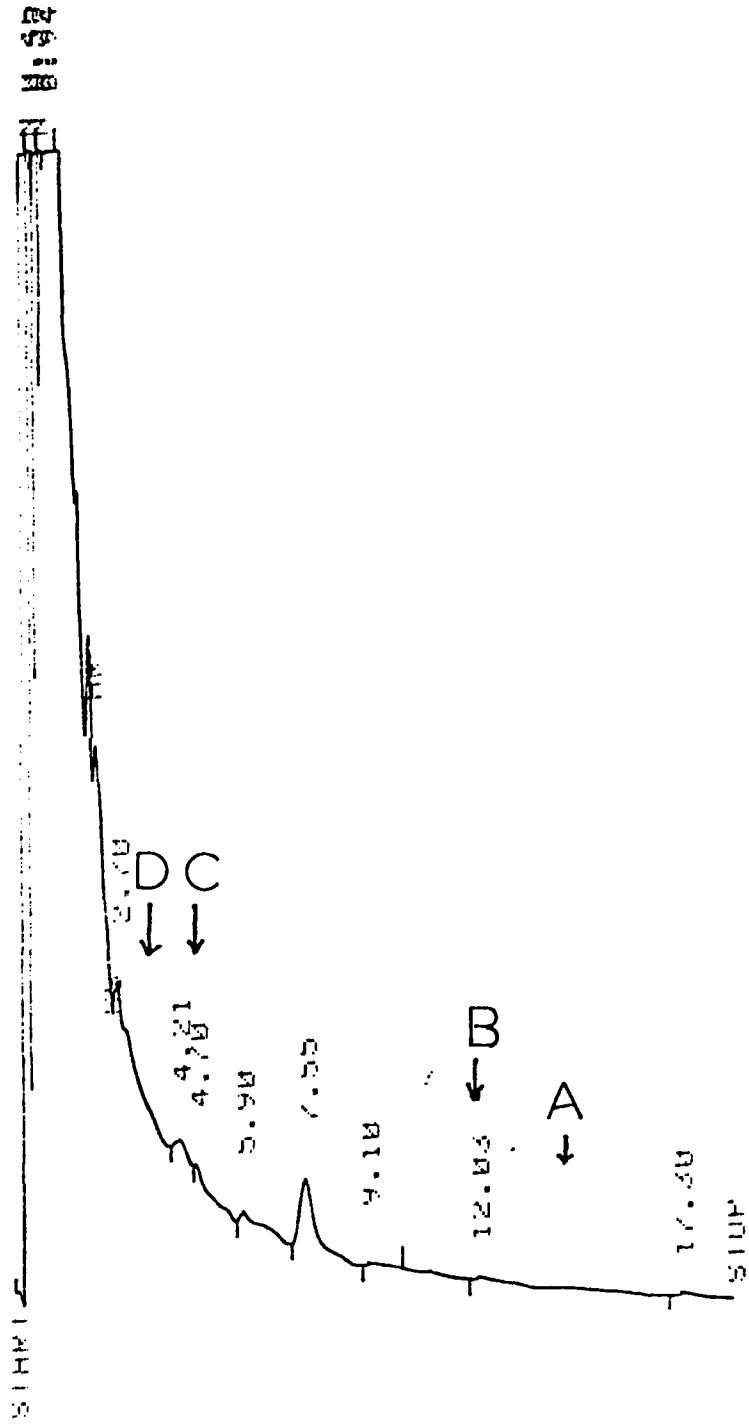
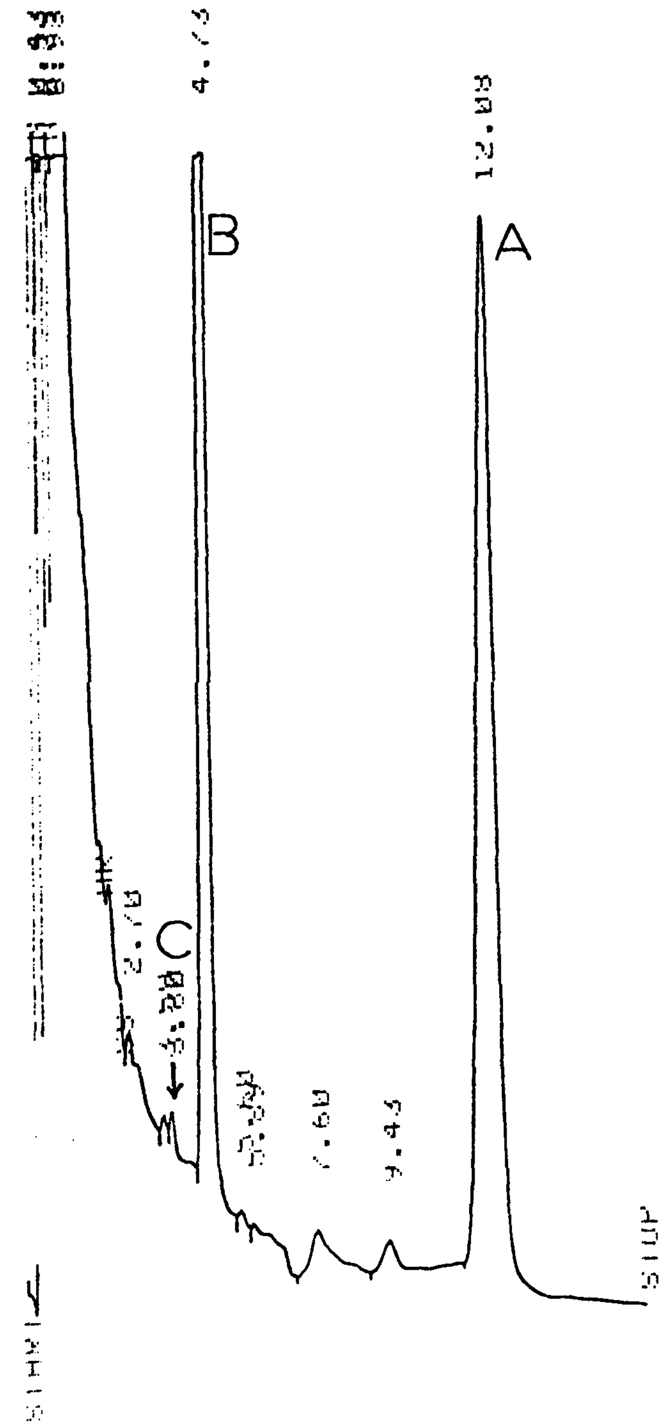

D
C
B
A
12.03
17.49

Figure 3

Chromatogram of swine plasma from a swine dosed with 1.2 mg/kg body weight T-2 toxin intravascularly. Blood was sampled 10 minutes after toxin administration: A, T-2 toxin; B, HT-2; C, Neosolaniol.


A
B
C

IV. Trichothecene Toxin Production

A. Objectives of this study are:

1. To prepare limited quantities of the metabolites of T-2 toxin, deoxynivalenol, and nivalenol for use as analytical standards.

2. To screen isolates of Fusarium for toxin producing potential and to identify and quantitate the toxins produced.

3. To screen fungal isolates and select a limited quantity of these cultures which produce appropriate toxins, i.e. isolates which produce a) T-2 and metabolites, b) T-2, DAS and metabolites, c) deoxynivalenol and metabolites and d) nivalenol and metabolites.

4. Fungal isolates selected will be cultured and crude extracts supplied to USRAMIID for laboratory animal toxicity testing. All batches will be analyzed for both types and quantities of toxins produced. The batches will be of sufficient size such that all small animal toxicity testing can be conducted from single production lots in order to ensure quality control.

5. All fungal isolates will be stored in several different manners in order to maintain cultures without mutation and to ensure reproductibility in toxin production.

B. Procedure

Several isolates of Fusarium which produce trichothecenes have been acquired. The isolate F. tricinctum NRRL 3299 has proven to be an excellent T-2 toxin producer which yields up to 3000 mg/kg T-2 toxin under appropriate conditions.

Fusarium tricinctum 3299 was cultured under 3 different conditions in order to produce extracts with different combinations of trichothecenes. The three different batches were produced as follows:

a) Erlenmeyer flasks containing 200 g corn grits were inoculated and incubated at room temperature for 7 days followed by 3 weeks at 15°C. The molded corn was then extracted 2 x 500 mL with methanol/water (9+1). An equal volume of water was added to the methanol extract followed by 2 partitions with 2 + 1 chloroform-ethyl acetate. The organic layer was dried with sodium sulfate and concentrated to a volume of 120 mL. This extract was designated CAI-82.

b) One hundred pounds of wheat was placed in a 30 gallon fermentor and inoculated with F. tricinctum 3299. The wheat was incubated at room temperature for 7 days followed by 30 days at 15°C. The wheat was then covered with methanol-water (9+1), stirred and allowed to stand 1 week. After filtration the wheat was extracted a second time with methanol-water (9+1). One half volume of 10% NaCl was added to the combined methanol extracts and the mixture partitioned two times with equal volumes of methylene chloride-ethyl acetate (2+1). The organic phase was split into two fractions, dried over sodium sulfate and concentrated. The two extracts were designated WAI-82 and WBI-82.

c) Fifty-five pounds each of cracked corn and corn grits were placed into a 30 gallon fermentor, inoculated with F. tricinctum 3299 and incubated at room temperature for 45 days. The culture was then extracted as described for wheat. One half of the extract was dried, concentrated and brought to a volume of 160 mL. This extract was labeled C30-1-83.

Quantitation of the extracts was accomplished by capillary gas chromatography with flame ionzation detection. Conditions were as follows: 22 m x 0.25 mmid x 0.25 micron fused silica column (DB 1701); flow rate 30 cm/sec helium; injector 250°; detector 300°. A 1 microliter sample was injected using the splitless injection technique at a column temperature of 175°. The column was held at 175° for 1 minute, programmed from 175-240° at 30°/minute and from 240-295° at 3.5°/minute. Additional capillary programs which can separate all known trichothecenes are under investigation. Sample extracts were derivatized to their corresponding TMS* ethers by adding 50 microliters TBT* and 150 microliters tetrahydrofuran (THF) followed by heating at 60°C for 10 minutes. Iso T-2 was added to the samples prior to derivatization as an internal standard. Under these conditions 50 ng of each standard compound (T-2 toxin and metabolites) produced peaks which were well separated and just off scale at an attenuation setting of 3.

Results

The predominant toxin in the extracts from the two cultures grown at 15°C was T-2 toxin whereas HT-2 was the predominant toxin in the culture grown at room temperature. See Table 1 for actual values. Diacetoxyscirpenol and neosolaniol were detected in all extracts. See

*TMS - Trimethylsilyl, TBT - TriSil TBT, Pierce Chemical Co., Rockford, IL

Figures 1 and 2 for chromatograms of standard trichothecenes and culture extract CAI-82, respectively. Additional peaks which may be trichothecenes were also observed in the chromatograms. The identification of these compounds is in progress. This work will be aided when our mass spectrometer becomes operational (summer of 1983).

Additional isolates of Fusarium are being acquired and screened for toxin producing potential including isolates reported to produce 1) doexynivalenol, 2) nivalenol and 3) fusarenone. We have also acquired several isolates of Stachybotrys which produce macrocyclic trichothecenes. All isolates are being stored under liquid nitrogen in order to maintain cultures without mutation and loss of toxin producing potential.

Several metabolites of T-2 toxin have been prepared for use as analytical standards including: HT-2, T-2 tetraol, T-2 triol, Acetyl T-2, Tetraacetate, Neosolaniol and 4-Deacetylneosolaniol. Relatively small amounts (5-100 mg) of these toxins are presently available due to the limited amount of T-2 toxin available for synthetic reactions at the beginning of this project. Synthesis of these metabolites will be repeated in the future with larger quantities of starting material (T-2) in order to provide greater quantities of purified trichothecene standards.

Deacetylneosolaniol (4-DN) was prepared by incubating T-2 toxin with rat liver homogenates. The resulting compound has been purified by column chromatography and HPLC. Additional incubations will be repeated with larger quantities of T-2 toxin in the future.

We have also prepared small amounts of purified neosolaniol from Fusarium culture extracts. Extraction and purification of additional neosolaniol is in progress.

Further work on HPLC conditions to optimize the separation and purification of trichothecene standards is in progress.

TRICHOTHECENES PRESENT IN CULTURE
EXTRACTS OF FUSARIUM TRICINCTUM 3299

TABLE IV-1

Lot #		Concentration (mg/mL)				
	volume (mL)	T-2	HT-2	Neo	DAS	TOL
CAI-82	120	78.22	2.36	5.43	1.49	ND
WAI-82	120	9.03	1.05	0.45	0.16	ND
WBI-82	160	2.58	0.45	0.16	0.06	.03
C30-1-83	160	1.30	3.80	0.22	0.17	0.05

Neo = Neosolaniol

DAS = Diacetoxyscirpenol

TOL = T-2 tetraol

Figure 1

Chromatogram of standard trichothecenes (TMS ethers) including: 1, Iso T-2 (Internal standard); 2, T-2; 3, HT-2; 4, Neosolaniol; 5, T-2 tetraol. Separation was accomplished on a 22 m DB-1701 fused silica capillary column.

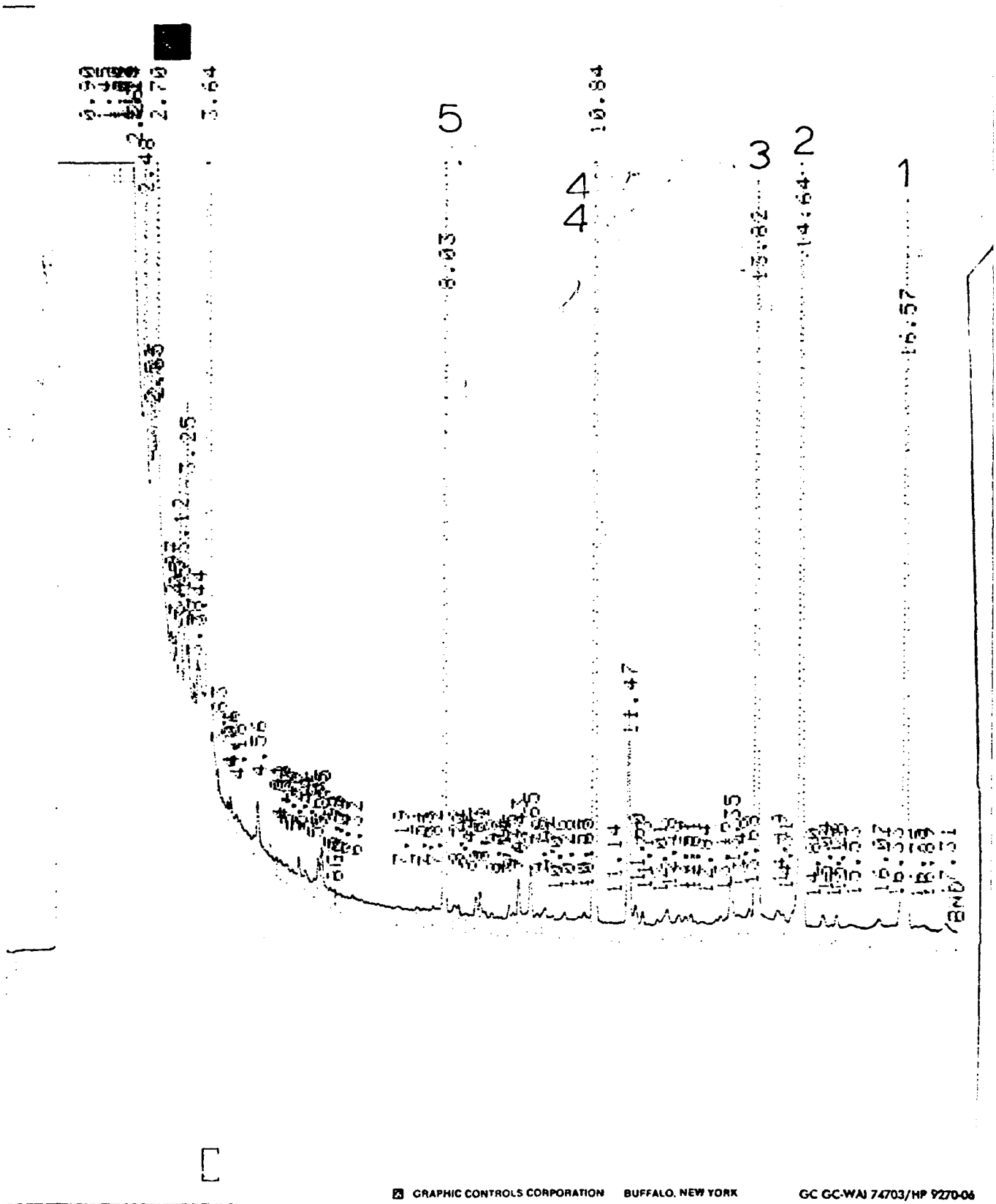


Figure 2

Chromatogram of extract CAI-82 (TMS ethers) on a 22 m DB-1701 fused silica capillary column. The following compounds were identified: 1, Iso T-2 (Internal standard); 2, T-2 toxin; 3, HT-2; 4, Neosolaniol; 5, Diacetoxyscirpenol.

1
2
3
4